TECHNICAL MANUSCRIPT 57

SINGLE-DOSE ASSAY TECHNIQUE FOR VARIOLA VIRUS

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U. S. ARMY CHEMICAL-BIOLOGICAL-RADIOLOGICAL AGENCY
U. S. ARMY BIOLOGICAL LABORATORIES
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Jean M. Riley
Michael D. Orlando

Process Development Division
DIRECTOR OF DEVELOPMENT

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ABSTRACT

The standard measurement for assay of the pox viruses is titration of pock-infectious units (PIU) on the chorioallantoic membrane (CAM) of embryonated eggs. Pock counts are not only time-consuming and imprecise but also do not always reflect the degree of animal infectivity. Thus a biological assay is needed that would (1) require a minimum of time and (2) have acceptable precision. Preliminary titrations made in 11-day-old embryonated eggs and in suckling mice (6 to 24 hours of age) demonstrated a linear relationship between the concentration of variola virus injected and the mean time to death (MTD) of the hosts. This linear response indicated the injection of a single dose of virus suspension and the measurement of the MTD should result in an acceptable assay method.

Seven replicate samples of liquid preparations (20 per cent CAM in heart infusion broth) and the freeze-dried material obtained with these suspensions were assayed in triplicate and the MTD values (in hours) with estimated 95 per cent confidence limits were:

Preparation	Eggs	Suckling Mice
Liquid	76 ± 18	181 ± 14
Dry	42 ± 6	107 ± 13

The use of the MTD assay resulted in a saving of time, a saving in the number of hosts necessary per assay, and increased precision.

SINGLE DOSE ASSAY TECHNIQUE FOR VARIOLA VIRUS

The standard method for assaying pox viruses is titration of pock-infectious units (PIU) on the chorioallantoic membrane (CAM) of embryonated eggs. Theoretically these pock counts should follow a Poisson distribution. However, a literature survey indicated that, regardless of the pox virus that was being investigated, the variance obtained in pock counting was far greater than expected for a Poisson distribution. Kaplan and Belyavin,[1] who made no adjustments in their assays of vaccinia virus, found that the variance of their pock counts was so wide that no estimate of the coefficient of variation was possible. In other cases, adjustments have been made in the counts to reduce the coefficient of variation to about 25 per cent. These adjustments were made by assigning double value to counts the investigator considered to be in the "normal" range[2] or by counting the pocks on only those membranes having no nonspecific lesions or encroachments of the albumen sac.[3] These adjustments resulted in considerable improvement in variance, but not enough to approach the variance expected with a Poisson distribution.

We needed a biological assay for the Yamada strain of variola virus that would (a) require a minimum of time and (b) have acceptable precision. Cabasso and Moore[4] found that an LD_{50} assay in eggs was feasible and of acceptable precision. These investigators also noted that there seemed to be a linear relation between the log-dilution injected and the reciprocal survival time of the embryo. In addition, several investigators[5-7] have recently found that suckling mice less than 24 hours of age were susceptible to variola virus. A close examination of their data indicated that there was also a linear relation of dose and time-to-death of the mice.* These observations offered the possibility of (a) an LD_{50} titration and (b) an assay based on the mean time-to-death (MTD) of a group of embryonated eggs or of suckling mice given a single dilution of virus.

Pock counts were made in the usual manner by dropping the CAM of 11-day embryonated eggs and inoculating 0.1 milliliter of appropriate dilutions of virus suspension on the dropped CAM. Ten eggs were inoculated for each dilution and the eggs were incubated at 35°C for 72 hours. When MTDs or LD_{50}'s were to be calculated, the CAM was dropped as for pock counts, but 0.25 milliliter of virus suspension was delivered on the CAM, and deaths were recorded daily for seven days.

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

Swiss-Webster albino mice ranging in ages from 6 to 24 hours were used. Litter sizes varied from 4 to 15 mice, so the litters were pooled and the mice were redistributed so that each mother was given eight young. The young were fed on the uninoculated mothers that were held in the cages with the suckling mice for the duration of the observation period. The mice were inoculated intracranially with 0.01 milliliter of appropriate dilutions from the series prepared for the egg assays. The observation period was 14 days and deaths were recorded daily.

In calculating the MTD for both eggs and mice the reciprocal transformation of Brownlee and Hamre[8] was used. Deaths discovered at successive check points were assumed to have occurred at random in the interval between observation periods, and the midpoint of the interval was taken as the best estimate of time of death. Thus, a host alive at one point and dead at the following was considered to have died at the hour halfway between the two observations. Hosts alive and well at the end of the observation periods were considered to have escaped infection. The LD_{50} value was calculated by the method of Reed and Muench.[9]

Preliminary titrations were made in embryonated eggs and in suckling mice to determine if a linear relationship existed between the viral concentration injected and the mean reciprocal time-to-death of the hosts. As shown in Figure 1, the relationship was linear in eggs, with curvature occurring at the 10^{-7} dilution, in which about 50 per cent survival of hosts occurred. Figure 2 shows that similar results were obtained with mice, although the slope is different. These linear responses indicated that both LD_{50} and single-dose assays were feasible in both embryonated eggs and suckling mice.

The LD_{50} values obtained in eggs and in mice are shown in Table I. There was no significant difference between the values obtained in eggs and those obtained in mice according to analysis of variance and F tests at the five per cent level of probability. Also, the inherent variability within each host was not significantly different.

A close correlation was obtained between the MTD of groups of mice inoculated with the 10^{-3} dilution and the calculated LD_{50} value (Figure 3). This indicates that a reference curve from which an estimated LD_{50} value could be obtained could be established rather easily from the MTD of a group of mice injected with a single dilution. However, limited animal holding space precluded the adoption of the LD_{50} titration for further work, so this measurement was discontinued.

From the preliminary titrations, the 10^{-3} dilution was selected as the most suitable for the single-dose assay, as it met the requirements of (a) assuring definition between death from trauma and death from the virus, yet assuring 100 per cent mortality, (b) completing an assay in eggs in about three days and an assay in mice in five to six days, and (c) reducing the need for observation of hosts to once daily.

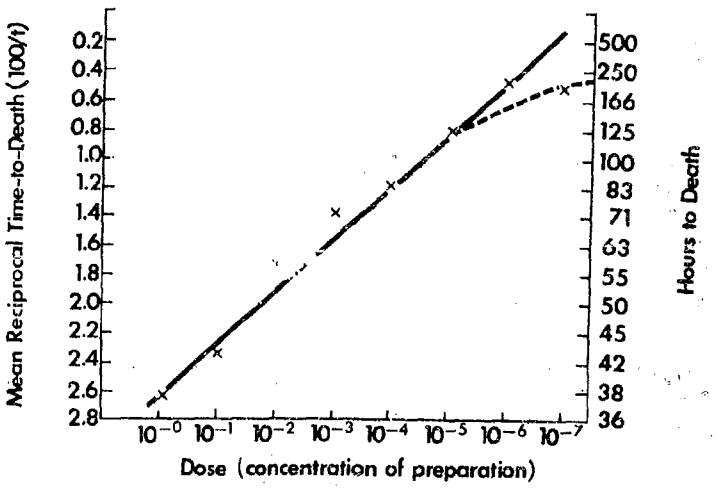

Figure 1. Relationship of Dose with Mean Time-to-Death for Eggs Inoculated with Variola Virus.

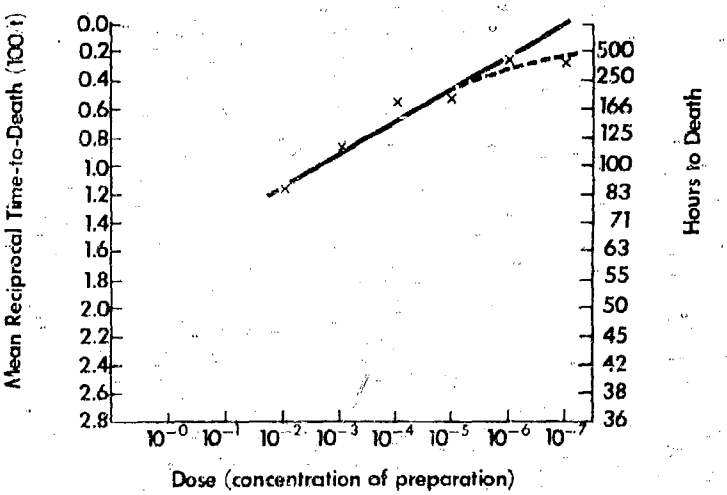

Figure 2. Relationship of Dose and Mean Time-to-Death for Suckling Mice given Variola Virus.

TABLE I. LD_{50} VALUES OBTAINED IN TWO HOSTS GIVEN VARIOLA PREPARATIONS

Type of Preparation		Host	
		Embryonated Eggs	Suckling Mice
1	Liquid	6.58	5.73
2	"	6.61	6.60
3	"	7.41	5.94
4	"	6.59	6.54
5	Dry	6.48	6.60
6	"	8.09	7.88
7	"	7.15	6.85
8	"	8.25	8.74
9	"	6.18	5.24

Seven replicate samples of a liquid preparation (20 per cent CAM in heart infusion broth) and of the dry material obtained from this liquid preparation were assayed in triplicate for pock infectious units and for MTD in eggs and in mice. When pocks were counted, no adjustments were made such as discarding membranes having nonspecific lesions. The values obtained are shown in Table II. The variance of the pock counts was far greater than expected from a Poisson distribution; the coefficient of variation obtained was 25 per cent with the liquid preparation and 61 per cent with the dry materials. This is not a true reflection of a difference in assaying liquid and dry materials because as work continued and the precision of the pock counts was followed, there was no difference in precision of pock counts from the two types of material. The variance within a sample was also greater than that from a Poisson distribution and the individual pock counts had an average coefficient of variation of about 40 per cent.

Variances obtained with the single-dose assay were all far below that expected from a Poisson distribution and coefficients of variation ranged from 5.2 to 13.6 per cent. The MTD values shown with their 95 per cent confidence limits were adopted as the basis for evaluating all experimental materials, each material being rated as better than, equal to, or poorer than the standard.

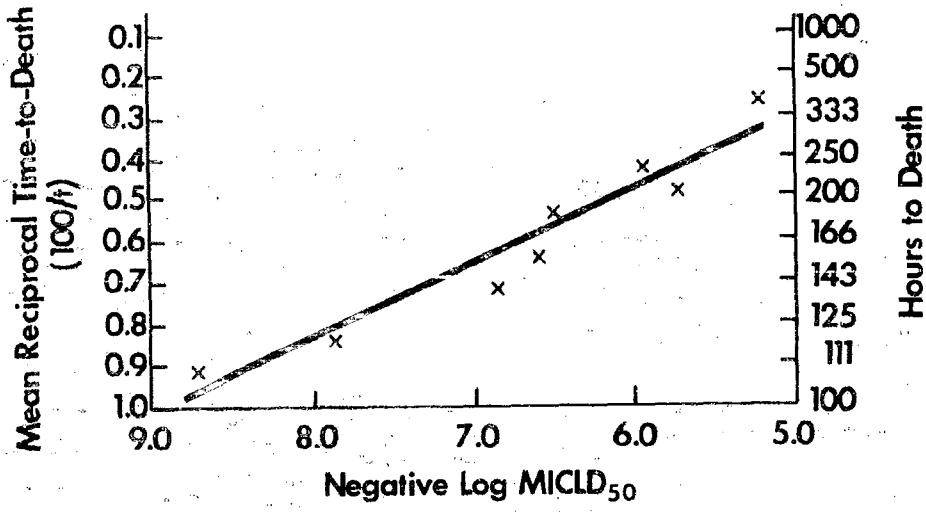

Figure 3. Relationship between LD_{50} Value and MTD of Suckling Mice Injected with Variola Virus.

TABLE II. COMPARISON OF ASSAY METHODS FOR VARIOLA VIRUS

		CV, Per Cent
Liquid Preparations		
PIU/ml	$1.8 \pm 0.56 \times 10^7$	25
Egg MTD, hr.	67 ± 7	8.2
Mouse MTD, hr.	183 ± 12	5.2
Dry Preparations		
PIU/ml	$8.9 \pm 4.7 \times 10^6$	61
Egg MTD, hr.	42 ± 6	13.6
Mouse MTD, hr.	107 ± 7	8.6

During our investigations we found that it was necessary to assay both in eggs and in mice. Treatment of both vaccinia virus and variola virus with certain chemicals, such as acetone or ether, resulted in materials with very high pock counts but with no ability to infect mice. Thus we used the assay in eggs as the routine measurement and the assay in mice as a control on infectivity.

The use of this single-dilution assay for variola has resulted in a biological measurement having about five times the precision of pock counts and at the same time has resulted in a saving of time and a reduction in the number of hosts required per assay.

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